Original paper

Comparison of mutation profile between primary phyllodes tumors of the breast and their paired local recurrences

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Phyllodes tumor of the breast (PTB) is a rare neoplasm and accounts for 0.2-2.0% of breast cancer in women. Histopathological diagnosis of the tumor is difficult, and histological features do not always predict the course of the disease and the risk of progression. Pathogenesis and molecular biological characteristics as well as PTB prognostic factors are unknown. In search for genetic factors affecting PTB progression, 10 patients were analyzed for whom material from the primary tumor and local recurrence was available. DNA isolated from paraffin blocks was sequenced using the next-generation sequencing method (NGS). In 4 pairs, consisting of primary tumor and local recurrence, probably pathogenic/pathogenic variants were detected, and in three pairs they were observed in the *CDKN2A* gene, while other variants were found in *PTEN* and *TP53* genes. NGS results indicate that the above-mentioned variants are hereditary, which suggests that the *CDKN2A* gene might be involved in cancerogenesis of PTB. Additionally, the selected pathogenic variant of *EGFR* gene was exclusively detected in one recuurent tumor, which might suggest the involvement of this gene in the mechanism of progression. In order to determine if this variant is associated with progression, the frequency of this mutation should be examined in larger group of malignant and borderline tumors.

Key words: phyllodes tumor, breast, next-generation sequencing, gene variants.

Introduction

Phyllodes tumor of the breast (PTB) is a rare neoplasm (it constitutes from 0.2 to 2.0% of all breast neoplasms in women), composed of elements of glandular epithelium, myoepithelium and stroma, which determines the biology of this cancer [1]. The World Health Organization classification distinguishes three PTB subtypes (benign, borderline and malignant) [2]. These subtypes present a morphological continuum from benign to malignant [1]. The classification is based on a combination of several histological features including: the degree of stromal atypia, stromal overgrowth, mitotic activity and type

of tumor border. For this reason, histopathological diagnosis of PTB is difficult and based only on histological features, it is not always possible to assess the course of the disease and the risk of cancer progression [3]. Currently, none of the biomarkers tested and described in the literature have been used in everyday clinical practice. Pathogenesis and molecular biological characteristics of PTB are unknown and no PTB prognostic factors have been identified. Generally, accepted treatment approach for patients with all of PTB types is breast conserving surgery with a margin >1 mm, which is sufficient to prevent local recurrence of PTB [4, 5]. There are no indications for resection of axillary lymph nodes or sentinel lymph node biopsy, because metastases to the lymph nodes occur very rarely [4, 6]. There are also no indications for hormonal therapy in patients with PTB. Adjuvant radiotherapy is recommended in borderline and malignant PTB, if the resection margin is less than 1 cm [4, 5, 7].

Most patients with diagnosed PTB have a good prognosis, with local recurrence risk from 17% in the benign type, up to 27% in the malignant type [8]. Distant metastases occur even in 22% of malignant PTB cases [8, 9]. The histological subtype of PTB is the only prognostic factor in these patients [6]. However, in some individuals, histological features do not allow for adequate assessment of the course of the disease (prediction of local recurrence, distant metastases or survival). This research has been undertaken to search for gene mutations related to the process of local recurrence in breast phyllodes tumor. If the onset of recurrence is caused by specific molecular changes, these changes should be present in most PTB recurrences and possibly in their respective primary tumors.

Material and methods

Patients

Between 1990 and 2013, 159 patients diagnosed with PTB were treated at the Oncology Center in Krakow. In this group, 16 patients developed local recurrence.

In 4 patients, after re-evaluation of pathology slides recurrent tumors were classified as sarcomas. Two patients were operated outside the Oncology Center and obtaining tissue material from the primary tumor was impossible. This pilot study was carried out on a group of 10 selected patients. There were 4 malignant primary PTBs (in one case there were two clearly separated components: benign and malignant), 2 borderline PTBs, 3 mixed types (benign/borderline) and 1 benign PTBs (Table I). In 4 cases progression was observed (Table I).

The study has been reported to the Ethical Committee at the Regional Medical Chamber in Krakow and received positive decisions (number: L.DZ.OIL/KBL/1/220/71220). This is a retrospective study using archived preserved tissues. There were no direct contact with patients, no modification of diagnostic or treatment procedures. None of personal patients' data were revealed and no specific patient consent was needed.

Next-generation sequencing

DNA was extracted from formalin-fixed paraffin-embedded tissues. Before extraction the tissue slides were evaluated by a pathologist to confirm the diagnosis and to select the paraffin blocks most suitable for molecular analysis. In the case of primary tumor with two clearly separated components (benign and malignant), for each component DNA was isolated separately. DNA was obtained from tissue sections using a semi-automatic method with Maxwell® RSC Instrument (Promega). The Maxwell® RSC DNA FFPE Kit (Promega) was used to isolate genomic DNA using paramagnetic particles. The amount of DNA was assessed fluorimetrically using the Qubit™ dsDNA HS Assay Kit and Qubit 3.0 device (ThermoFisher Scientific). Next-generation sequencing (NGS) was performed on IonTorrent platform. Two hundred and seven regions in 50 genes that are most often mutated in solid tumors were sequenced with Cancer Hotspot Panel v2. Libraries for sequencing were prepared automatically on Chef instrument using Ion Ampliseq™ Cancer Hotspot Panel v2 Chef-Ready Kit. Sequencing was performed using S5 instrument, Ion 510™ & Ion 520™ & Ion 530™ Kit and Ion 530™ Chip Kit. S5 Torrent Server Torrent Suite 5.10.1 software was applied for coverage analysis and to identify genes variants. Results (vcf files) were annotated using wANNOVAR (http://wannovar.wglab.org). Variants for which satisfactory sequencing conditions were obtained and which are estimated to occur in the population with a frequency below 1% were qualified for further analysis. Variants occurring in the population with a frequency above 1% are classified as polymorphisms. With the help of the Varsome (https://varsome.com) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) databases variants were classified into 5 classes: pathogenic, probably pathogenic, of unknown significance, probably benign and benign. The workflow of the experiment is presented in Figure 1.

Table I. Quality of obtained NGS data

Pair no.	Sample number and description	Age of block (years)	Mean read length	Mapped reads	Percent reads on target	Average base coverage depth	Uniformity of base coverage	Target base coverage at 100× (%)	Number of detected variants
1	M5 PT benign/ borderline	17.0	99 bp	979 151	**93.64%**	3857	97.52%	**100**	243
	M6 R(1st)* benign/ borderline	13.8	103 bp	991 806	95.95%	4106	96.89%	100	344
2	M7 PT borderline	13.1	106 bp	1 288 888	97.57%	5517	99.48%	100	30
	M8 R(3rd) borderline	11.6	113 bp	1 383 908	98.68%	6250	99.48%	100	22
3	M9 PT benign	10.2	107 bp	1 254 256	98.32%	5453	98.25%	100	47
	M10 R(1st) malignant	7.2	115 bp	1 387 224	98.81%	6365	99.38%	100	22
4	M11 PT benign/ borderline	24.3	89 bp	149 326	70.56%	408.8	73.29%	68.88	858
	M12 R(2nd) malignant, sarcoma like G3	23.7	96 bp	493 945	86.83%	1734	88.23%	97.73	1211
5	M13 PT benign/ borderline	16.8	103 bp	1 008 257	97.05%	4217	97.50%	100	331
	M14 R(3rd) malignant	13.6	**107 bp**	1 430 807	98.77%	6260	87.01%	100	41
6	M15 PT borderline	17.7	**95 bp**	502 980	85.67%	1748	88.14%	100	987
	M16 R(2nd) malignant	7.50	111 bp	1 268 905	98.60%	5679	99.48%	100	18
7	M17 PT c. benign**	10.7	**112 bp**	1 165 877	**98.41%**	5271	99.48%	100	17
	M18 PT c. malignant	10.7	**114 bp**	909 468	98.21%	3997	99.48%	100	19
	M19 R(1st) malignant	10.5	**104 bp**	1 088 939	96.62%	4569	99.17%	100	89
8	M20 PT malignant	6.8	**106 bp**	1 353 842	98.45%	5859	87.84%	100	55
	M21 R(1st) malignant	6.6	**109 bp**	1 371 358	98.70%	6070	89.48%	100	39
9	M23 PT malignant	6.6	**113 bp**	1 385 681	98.67%	6309	98.84%	100	17
	M24 R(1st) malignant	5.6	**109 bp**	1 295 685	98.49%	5714	86.35%	100	43
10	M25 PT malignant	24.7	**126 bp**	5 510	33.47%	7.02	65.13%	0.21	17
	M26 R(1st) malignant	24.2	**133 bp**	148 752	65.42%	382.3	75.63%	69.03	904

**– the number of tested recurrence, ** c – component, PT – primary tumor, R – recurrence*

Results

Assessment of NGS data

Next-generation sequencing (NGS) was performed for 10 pairs consisting of primary and recurrent tumor. The most important parameters of the run are presented in Table I. The age of the blocks ranged from 5.6 to 24.7 years. For all samples there was enough of DNA to perform sequencing. However, in the case of two pairs with the oldest blocks (aged 23-24 years) the data obtained were of low quality and mutation analysis for these samples was not possible (Table I). DNA from these samples was too fragmented, especially from primary tumor.

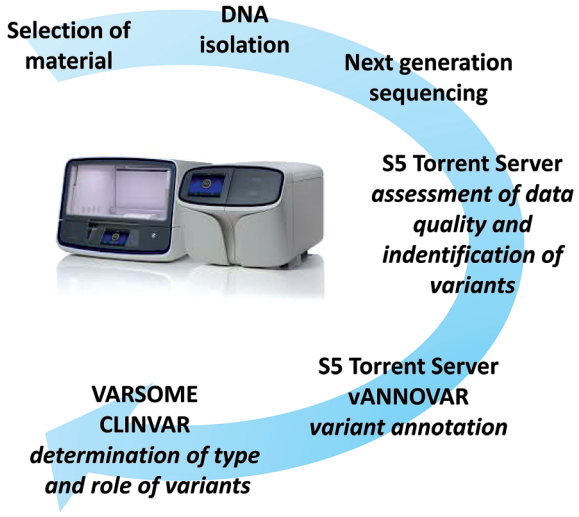

Fig. 1. Workflow of the experiment and data analysis

These data were characterized by too low number of reads, low uniformity (65-88%), percent reads on target (33-86%) and low target base coverage at 100× (i.e. the percentage of bases for which 100 readings were obtained, the recommended minimum required for analysis is 98%) (Table I). For all other cases, coverage at 100× was 100%, so even for 17-year-old blocks it was possible to obtain material that allowed analysis. However, it should be noted that for blocks aged 13-17 years a large number of observed changes should be considered as sequencing errors (low sequencing parameters) and changes typical for the formalin-fixed material (change G>A and T>C).

Comparison of primary and recurrent tumor

Eight primary tumor – recurrence pairs (16 samples) were qualified for the analysis of mutation status. In 4 out of 8 pairs, probably pathogenic/pathogenic variants, according to Varsome and/or ClinVar databases, were detected. In three of them mutations were found in *CDKN2A* gene (Table II). In two pairs, the same variant creating the STOP codon and causing synthesis of the truncated protein was detected. The other variant in *CDKN2A* gene was a non-synonymous change, resulting in insertion of another amino acid. In the case of the *PTEN* gene, there was a change of the reading frame, resulting in truncated protein, while in the *TP53* gene non-synonymous change was found (Table II). Moreover, in one patient, two clearly separated components: benign and malignant were identified in the primary tumor. Both components were isolated and tested separately. Pathogenic/probably pathogenic variants in the *CDKN2A* and *TP53* genes were detected only in the malignant component. The same mutations were also detected in the recurrent tumor. Only in one pair, a mutation that was not present in the primary tumor (involving the *EGFR* gene) was detected in the recurrence.

Discussion

Breast phyllodes tumor is a rare breast neoplasm and most patients with diagnosed PTB have a good prognosis. Metastatic disease is observed less frequently than local recurrences, however, it is associated with worse prognosis [10]. Recurrences of malignant PTB are found in 23-30% of patients, while in benign and borderline PTBs recurrences appear in 10-20% and 14-25% of cases, respectively [1]. The frequency of local recurrence is higher in patients with malignant and borderline PTB than in benign cases [9]. Breast conserving therapy is proposed in all cases of local PTB recurrences, if a margin of healthy tissue can be achieved with good cosmetic result [11]. Benign PTB is successfully treated with surgery alone. In borderline and malignant type, with a tumor-free margin < 1 cm (0.3-0.8 cm), adjuvant radiotherapy is recommended [4]. Only in the cases of extensive recurrences, simple mastectomy may be necessary to obtain a negative margin.

In our study, we discovered mutations in primary tumour and also in matched local recurrence in *CDKN2A* (in 3 pairs), *PTEN* (1 pair) and *TP53* (1 pair) genes. Additionally, in one pair only in the recurrent lesion mutation in the *EGFR* gene was observed. According to COSMIC database (https://cancer.sanger.ac.uk/cosmic) a frequently mutated gene in

Table II. List of detected pathogenic or likely pathogenic variants according to Varsome and/or ClinVar databases

Paired sample numbers	Pathogenic or likely pathogenic variants
M7 PT borderline/ M8 R(3rd) borderline	primary tumor and recurrence: CDKN2A, NM_000077.4:c.238C>T (p.Arg80Ter)
M9 PT benign/ M10 R(1st) malignant	primary tumor and recurrence: CDKN2A, NM_000077.4:c.251A>G (p.Asp84Gly)
M20 PT malignant/ M21 R(1st) malignant	recurrence: EGFR, NM_005228.5:c.2297T>C (p.Met766Thr)
M23 PT malignant/ M24 R(1st) malignant	primary tumor and recurrence: PTEN, NM_000314.8:c.170dupT (p.Leu57PhefsTer6)
M17 PT c. benign**/ M18 PT c. malignant/ M19 R(1st) malignant	primary tumor benign component – lack of variants primary tumor (malignant component) and recurrence: CDKN2A, NM_000077.4:c.238C>T (p.Arg80Ter) TP53, NM_000546.5:c.711G>A (p.Met237Ile)

**– the number of tested recurrence, ** c – component, PT – primary tumor, R – recurrence*

phyllodes tumours is *MED12* (55%). Unfortunately, Cancer Hotspot Panel v2 does not asses mutation in *MED12* gene. However, mutations of *MED12* gene were less frequently observed in malignant phyllodes tumors than in fibroadenomas, benign and borderline phyllodes tumors [1]. Moreover, the presence of *MED12* mutation was associated with longer disease-free survival, whereas its absence was related to a higher likelihood of recurrence [1].

In our study, we observed pathogenic variants in *PTEN*, *TP53*, *EGFR* and *CDKN2A* genes. The first three of them are reported by COSMIC in "Top 20 genes" for phyllodes tumours: *TP53* (mutation in 12%), *EGFR* (6%), *PTEN* (4%). In our study, most frequently mutated gene was *CDKN2A*, in which mutation were observed both in primary tumors and local recurrences. Mutations or homozygous deletions in *CDKN2A* gene in phyllodes tumour are also reported by other authors [12, 13, 14]. Tan *et al.* studied a group of 20 cases of phyllodes tumour, including 11 cases with no recurrence and 9 with local recurrence/metastases. In this group, homozygous deletion at 9p21 involving *CDKN2A* gene was reported in two cases with progression, while in two other cases with progression single copy loss at 9p21 was noted. None of these aberrations was observed in cases that did not develop recurrences [13]. In our study, we detected pathogenic variants in the *CDKN2A* gene in as many as three out of eight pairs of primary and recurrent tumors. Tan *et al*. and our findings may suggest that *CDKN2A* gene is involved in the development of primary PTB and local recurrences/metastases.

It is worth to mention that all mutations detected in the paired samples affected the suppressor genes:

- *CDKN2A* – encodes a protein that is a CDK4-dependent cyclin kinase inhibitor and a P53 stabilizing protein,
- *PTEN* – encodes a protein that negatively regulates the PI3K/AKT signaling pathway,
- *TP53* – encodes a protein involved in, among others, a mechanism of controlling cell arrest in the cell cycle, apoptosis and DNA repair.

The allelic frequency of variants detected in the sequenced material from the primary tumor and recurrence (40-60%) suggests that they might be hereditary changes. In order to confirm this hypothesis in the future research, an attempt will be made to assess the presence of the detected variants in archival material derived from normal tissue (from the paired samples) by Sanger sequencing.

Only in one pair, in the recurrence but not in the primary tumor, pathogenic variant in the *EGFR* gene was found. This might be a possible molecular hallmark of progression. In order to determine if this variant is associated with progression, the frequency of this mutation should be examined in a larger group of malignant and borderline tumors.

Conclusions

The *CDKN2A* gene might be involved in the development of phyllodes tumor of the breast and its local recurrences/metastases.

Mutations in the *EGFR* gene might be a possible molecular hallmark of progression.

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